JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
60th Anniversary

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • Publication Date (Web): 15 Nov 2012

Just Accepted

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Generation of the Volatile Spiroketals Conophthorin and Chalcogran by Fungal Spores on Polyunsaturated Fatty Acids Common to Almonds and Pistachios

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ABSTRACT

The spiroketal (*E*)-conophthorin has recently been reported as a semiochemical of the navel orangeworm moth, a major insect pest of California pistachios and almonds. Conophthorin and the isomeric spiroketal chalcogran are most commonly known as semiochemicals of several scolytid beetles. Conophthorin is both an insect- and plant-produced semiochemical widely recognized as a non-host plant volatile from the bark of several angiosperm species. Chalcogran is the principal aggregation pheromone component of the six-spined spruce bark beetle. Recent research has shown conophthorin is produced by almonds undergoing hull-split and both spiroketals are produced by mechanically damaged almonds. To better understand the origin of these spiroketals the volatile emissions of orchard fungal spores on fatty acids common to both pistachios and almonds were evaluated. The volatile emission for the first 13 days of spores placed on a fatty acid was monitored. The spores investigated were *Aspergillus flavus* (atoxigenic), *A. flavus* (toxigenic), *A. niger*, *A. parasiticus*, *Penicillium glabrum*, and *Rhizopus stolonifer*. The fatty acids used as growth media were palmitic, oleic, linoleic, and linolenic. Spores on linoleic acid produced both spiroketals, on linolenic acid produced only chalcogran, and on palmitic and oleic acid did not produce either spiroketal. This is the first report of the spiroketals conophthorin and chalcogran from a fungal source.

KEYWORDS: *Aspergillus*, chalcogran, conophthorin, fatty acid, germination, *Penicillium*, *Rhizopus*, spiroacetal, spiroketal, spore, volatile

INTRODUCTION

The structurally simple spiroketals conophthorin, **1**, and chalcogran, **2**, (Figure 1) play an important role in numerous plant and insect chemical communication systems, particularly with respect to scolytid beetles and associated non-host plants.[1-3] Conophthorin is produced primarily as the *trans*-isomer by several plants from varying families as well as by numerous insects such as angiosperm and conifer scolytids, wasps, and the fruit fly, among others.[1] Chalcogran was first reported[4] as an aggregation pheromone from the six-spined spruce bark beetle in 1977 and largely remains an insect-produced semiochemical.

Despite their isomeric structures and similar semiochemical behavior there are few instances of concurrent production of conophthorin, **1**, and chalcogran, **2**, by a host source. Both spiroketals have been detected in the stems of willow,[5] from tropical orchid,[5] in the stems of aspen,[6] and more recently from bacteria.[7] During the same time period our laboratories have detected the spiroketals conophthorin and chalcogran from the *ex situ* volatile evaluation of mechanically damaged almonds[8, 9] as well as conophthorin from the *in situ* collection of volatiles from almonds at hull split.[10] It is notable that the emission of conophthorin and chalcogran from almond as a host plant diverges from the heretofore-reported characteristic origins. In their 2008 report[9] on damaged almond emissions the authors noted the presence of volatiles typically associated with fungal growth but did not consider more thoroughly the genesis of the noted spiroketals.

Fungi are ubiquitous in California almond, pistachio, and walnut orchards. Among the most prevalent are species of *Aspergillus*, *Rhizopus*, and *Penicillium*.[11] *Aspergillus flavus* and *A. parasiticus* are a major food safety concern due to the production of hepatotoxic and carcinogenic aflatoxins.[12] Recent research in our laboratories has focused on the volatile

emissions of various almond orchard fungi and their possible correlation to aflatoxin contamination,[13] styrene production,[14] and relationship to the insect pest navel orangeworm.[15] However, these studies did not report the presence of either conophthorin or chalcogran despite almond as the host plant. To address this question our laboratories undertook an affiliated investigation of *ex situ* pistachio and almond volatile emissions over the course of a growing season to determine the origin of spiroketals reported in previous studies.[9, 10] The results of this investigation suggested we turn our attention to the emission of germinating spores. A search of the literature revealed several reports of volatile production by resting and germinating *Penicillium* spores,[16, 17] particularly 2-alkanones, which were observed on several instances when fatty acids were available.[13] Since the fatty acid content of both pistachios and almond are similar,[18, 19] and both tree nuts are affected by the insect pest navel orangeworm, the decision was made to study the volatile emissions of fungal spores on fatty acids that are common to pistachios and almonds.

The objective of this study was to establish if the spiroketals conophthorin and chalcogran were generated from the investigated fungal spores and given a positive result from this objective, to then determine: (a) whether the host carbon source for spores affect spiroketal production; (b) if the tested spores produce different amounts of spiroketals relative to each other; and, (c) if there is an optimal spore age for spiroketal production.

MATERIALS AND METHODS

Chemical Sources. The isomers of (*E*)-conophthorin and (*Z*)- and (*E*)-chalcogran were authenticated by comparison of retention times and fragmentation patterns to commercially available samples (Contech, Victoria, BC). Similarly, (3*Z*)-hexen-1-ol (Bedoukian Research Inc.

Danbury, CT) and 1-hexanol (Sigma-Aldrich, St. Louis, MO) were used for authentication, and the following carbon sources: palmitic and oleic acids (Calbiochem, Fisher Scientific, Pittsburgh, PA); linoleic and linolenic acids (Acros, Fisher Scientific, Pittsburgh, PA).

Preparation of fungal spores. Spores were isolated from fungal cultures obtained as pure strains from the ARS Culture Collection (NRRL). Spores from stock solutions of *Aspergillus flavus* (atoxigenic) (NRRL 18543), *A. flavus* (toxigenic) (NRRL 25347), *A. parasiticus* (NRRL 5862), *A. niger* (NRRL 326), *Penicillium glabrum* (NRRL 766), and *Rhizopus stolonifer* (NRRL 54667) were inoculated onto Petri dishes containing potato dextrose agar (PDA) and incubated at 30 °C for 13 days. Spore suspensions for each fungal isolate were prepared by irrigating the PDA cultures with 0.05% Tween 80 (Sigma-Aldrich, St. Louis, MO) and the spore concentration measured using a hemacytometer. Measured aliquots of the spore suspensions were filtered through 47 mm, 0.65 µm mixed cellulose ester membrane filters (Millipore) to collect, in triplicate, 0.9 – 1.0 x 10^9 spores for each fungal isolate (2.5 x 10^8 spores for *Rhiozopus stolonifer*). The underside of each triplicate membrane filter with spores was coated with 50 mg of an individual fatty acid that had been weighed into a 125 mL Ball jar. Water, 200 µL, was dispersed equally and drop-wise to the topside of each spore/membrane filter. Control treatments were prepared in identical collection containers and consisted of: 1) spores on membrane filters with no fatty acid treatment; 2) membrane filters with no spores treated with individual fatty acids; and 3) membrane filters with Tween wash and no spores or fatty acid. The collection containers were capped with a modified lid to allow for volatile sampling and incubated at 30 °C for 13 days. All treatments were performed in triplicate. Spores were assumed to have undergone

transition from resting to germination over the course of the experiment and under conditions provided.[20, 21]

Collection of volatiles. Volatiles were allowed to collect in a closed system prior to adsorbing onto 100 μm solid phase microextraction (SPME), polydimethylsiloxane fibers (Supelco, Bellefonte, PA). Individual collections were standardized using P.E.S.T. volatile collection parameters.[13, 22] The volatile permeation time, P, varied for the days analyzed (corresponding to spore age): d = 1, P = 24 h; d = 6, 8 and 13, P = 48 h; d = 4 and 11, P = 72 h; exposure time of the fiber to the collected volatiles, E = 20 min at 30 ˚C; storage time of the adsorbed volatiles on the fiber, S < 1 min; and volatiles were thermally desorbed, T = 6 min. The volatile collection chambers comprised a 125 mL Ball jar with lids fitted with a Teflon septum SPME port and a venting port. After SPME adsorption of the volatiles on d = 1, 4, 6, 8, and 11 the headspace of the jars were gently vented with 125 mL of air via a glass 250 mL syringe and through a sterile Millipore Millex-GP 0.22 μm filter. Filter paper was placed on the vent tube to ensure no loss of spores. The collection chambers were maintained at 30 ˚C during storage and SPME volatile collections.

Analysis of volatiles. Collected volatiles were thermally desorbed onto a DB-Wax column 60 m × 0.32 mm i.d. × 0.25 μm (J&W Scientific (Folsom, CA) installed on a 6890 gas chromatograph (GC) coupled to HP-5973 mass selective detectors (MS; Palo Alto, CA). Desorbed volatiles were analyzed with the following method: injector temperature, 200 ˚C; splitless mode; inlet temperature, 200 ˚C; constant flow, 3.1 mL/min; oven settings, initial temperature, 70 ˚C; hold time, 0.0 min; ramp 1, 5 ˚C/min to 125 ˚C; hold time 0.0 min; ramp 2, 30 ˚C/min to 200 ˚C; hold

time, 1.0 min; ramp 3, 30 ˚C/min to 230 ˚C; hold time, 2.5 min; final temperature, 230 ˚C; hold time, 1 min. MSD parameters: source temperature, 230 ˚C; MS source temperature, 150 ˚C; EI mode, 70 eV; solvent delay, 2.5 min; scan group 1, 40-350 *m/z*; scan group 2 at 20 min, 40-450 *m/z*. NIST, Wiley, and internally generated databases were used for fragmentation pattern identification. The identities of key volatiles were verified by injection of authentic samples and comparison of retention times and fragmentation patterns.

Statistics. All Statistical operations were performed in SigmaStat 3.1 (Systat Software, Inc., Chicago, IL). Total area counts for the individual volatiles (*E*)-conophthorin, (*Z*)- and (*E*)-chalcogran, 1-hexanol, and (3*Z*)-hexen-1-ol (analyzed components) were summed for each replicate (n=3) and time point (n=7) per isolate on each carbon source. Total area counts of each volatile were log-transformed for statistical analysis. One-way ANOVA was performed comparing individual spiroketal production between and within isolates. A P-value of < 0.05 was considered to be statistically significant after a post-hoc test of significance using a Bonferroni correction. Two-way ANOVA comparing log transformed area counts of the analyzed components was performed where spore age and isolate were the two factors for each isolate with a post-hoc test of significance using a Bonferroni correction. A P-value of < 0.05 was considered to be statistically significant. Pearson correlation analysis was performed with relative area counts of the analyzed components [# of measurements (21) = time points (7) × replicates per isolate (3)]. A P-value of < 0.05 was considered to be statistically significant. All graphs and tables show relative area counts.

RESULTS AND DISCUSSION

The objective of the investigation was clearly met with all tested fungal spores demonstrating the ability to produce detectable amounts of both spiroketals in addition to other fungal-associated and fatty acid breakdown volatiles. The control experiments evaluating the volatile emission of fungal spores only and fatty acids only did not produce any spiroketals. The control experiments with the various fatty acids did produce fatty acid breakdown products typical of each fatty acid.[9, 14] These volatile products along with other typical fungal-associated volatiles were detected; however, this report will focus discussion on the emission of the spiroketals from the tested spores.

Spiroketals from linoleic and linolenic acid

When placed in the presence of linolenic acid, all fungal spores emitted both the (*Z*)- and (*E*)-diastereomers (7.70 min and 7.78 min retention times, respectively) of chalcogran. The enantiomeric composition of the detected signals was not evaluated. In contrast, when linoleic acid was the available carbon source (*E*)-conophthorin, **1**, unknown enantiomeric composition (6.54 min retention time), and both diastereomers of chalcogran were detected. The fungal spores did not produce any detectable levels of spiroketals when allowed to develop in the presence of either oleic or palmitic acid.

When placed on linoleic acid the fungal spores produced all three spiroketals, but in varying amounts. Figure 2 illustrates the differences in spiroketal amounts produced by the spores. Total individual spiroketal production differed among the fungal spores over the 13 days of volatile evaluation ($P < 0.001$). Figure 2A illustrates spiroketal formation from the noted fungal spores on linoleic acid. *Aspergillus flavus* (toxigenic) produced equal amounts of the chalcogran

isomers when compared to *A. flavus* (atoxigenic) and *Penicillium glabrum*, but produced significantly greater amounts of both chalcogran isomers relative to *Rhizopus stolonifer*, *A. niger* and *A. parasiticus* ($P < 0.001$). With the exception of *A. niger* all spores tested produced numerically greater amounts of (*E*)-chalcogran (means of total compared) in a 1:1.3 *Z*:*E* ratio (s.e. +/- 0.05) when placed on linoleic acid. However, only *A. flavus* (atoxigenic) emitted a statistically significantly greater amount ($P = 0.03$ Bonferroni t-test after one-way ANOVA) of the (*E*)-chalcogran isomer. The remaining fungal spores produced statistically equivalent amounts of the (*E*)- and (*Z*)-isomers. This slight difference in chalcogran isomers corresponds well, albeit a little lower, when compared to the noted synthetic mixture *Z*:*E* ratio of 1:1.6 by GC-MS and 1:1.5 by GC-FID. The commercial synthesis of the two isomers slightly favors the more stable (*E*)-isomer (personal communication, Contech personnel).

The amount of conophthorin produced by the spores on linoleic was not as varied (Figure 2A). The fungal spores of *Aspergillus flavus* (both atoxigenic and toxigenic), *A. niger*, *Penicillium glabrum*, and *Rhizopus stolonifer* produced statistically equal amounts of conophthorin and significantly greater amounts of conophthorin than *A. parasiticus*. A few notable observations from *A. niger* were: it produced equal amounts (nearly identical relative abundances) of chalcogran isomers ($P = 1$ pairwise comparison) relative to the observed differences between the (*Z*)- and (*E*)-isomers from the other fungal spores; and, it was the only fungal spore to provide a higher total relative abundance of conophthorin than chalcogran ($P =$ 0.001 pairwise comparison of conophthorin to both isomers of chalcogran). This particular result is consistent with our previous investigation,[9] which determined the volatile emissions from *ex situ* almonds and included the detection and emission of both chalcogran and conophthorin. The

relative amounts of conophthorin and chalcogran were not provided in the investigations of conifers.[6, 7]

For spiroketal emission from spores developing on the triene linolenic acid (Figure 2B) the most obvious difference compared to spore germination on the diene linoleic was the absence of conophthorin. Unlike the emissions from spores on linoleic, the total emission of the chalcogran isomers were similar among the tested fungal spores with *A. flavus* (toxigenic) producing greater total emissions (numerically) of both chalcogran isomers (Figure 2B). The detected *Z*:*E* chalcogran isomer ratios were better resolved and except for *A. flavus* (toxigenic) were significantly different ($P < 0.010$) and with an average *Z*:*E* of 1:1.4 (s.e. +/- 0.02). Unfortunately, the spores of *Rhizopus stolofiner* were not easily grown and thus this particular fungal spore was not evaluated on linolenic acid. This absence of conophthorin from spores on linolenic acid led to the questioning of the role of the fatty acid during the biosynthesis of the spiroketals.

Spiroketal formation as a function of spore age and identity

Production of the individual spiroketals differed by spore age × spore identity ($P < 0.001$) with each main effect showing significance ($P < 0.001$). Figures 3A-F show the detected amounts of the spiroketals as a function of time. The data points correspond to the days volatile emissions were monitored by SPME GC-MS. Chalcogran isomers were detected on day 4 and detected for the remainder of the experiment. In contrast, conophthorin production was not detected until day 6 in all spores except for *A. parasiticus*, which showed conophthorin production on date 8 and then only in relatively small amounts (Figures 3A-B). Some observations of particular interest are first, though the overall mean spiroketal amount between *A. flavus* atoxigenic and toxigenic were statistically equivalent (Figure 2), the graphs in Figures

3A, 3C, and 3E showed that the trends of spiroketal production over time differed between these two fungal spores. For example, in *A. flavus* (atoxigenic) all three spiroketals peak in their production on day 8 followed by a rapid decrease in days 11 and 13, whereas spiroketal production in *A. flavus* (toxigenic) is identical to that in *A. flavus* (atoxigenic) on day 6 but instead of peaking and dropping the spiroketal production in *A. flavus* (toxigenic) stays relatively constant through all days of analyses. Second, though production of conophthorin from *A. niger* spores has a moderate start on day 6 and stays relatively low in day 8, conophthorin emission then appears to undergo rapid emission on days 11 and 13. Third, *Penicillium glabrum* fungal spores on linoleic acid provide overall emission patterns similar to the *A. flavus* strains with all three spiroketals starting with low emissions, spiking, and followed by a drop in emission prior to the end of the experiment (Figures 3B, 3D, 3F). Fourth, of all the fungal spores tested *Rhizopus stolonifer* emitted the most consistent relative amounts of all three spiroketals.

As noted earlier, all fungal spores only produced the (*Z*)- and (*E*)-chalcogran isomers when placed on linolenic acid (Figures 4A-B). In contrast to spores on linoleic acid, the emission patterns of the chalcogran isomers were fairly consistent within all tested spores with no dramatic spiking of either chalcogran from the individual fungal spores, with the exception of *A. flavus* (atoxigenic). Overall, the individual spores appear to emit more spiroketals during the period of time between day 6 and day 11. Visual inspection of the spores during the experiments revealed no detectable growth of mycelia. By conclusion of the experiments and for all isolates tested, fungal development was limited to sparse, un-branched hyphae visible only under magnification (32x dissecting scope).

Spiroketal biosynthesis

In addition to the noted disparity of spiroketal formation between the carbon sources, the two volatiles 1-hexanol and (3*Z*)-hexen-1-ol were observed via GC traces to be associated with conophthorin and chalcogran formation on linoleic and linolenic acids, respectively. It was noted that large amounts of 1-hexanol was formed when the fungal spores were placed on linoleic acid, yet no (3*Z*)-hexen-1-ol was detected. However, in the presence of linolenic acid the fungal spores emitted relatively large amounts of (3*Z*)-hexen-1-ol and only briefly generated 1-hexanol in ca. 10% of the relative abundance of (3*Z*)-hexen-1-ol.

The data in Table 1 and Table 2 provide the Pearson correlations for the emission of the spiroketals, 1-hexanol, and (3*Z*)-hexen-1-ol on linoleic and linolenic acid, respectively. For the fungal spores on linoleic acid (Table 1) there are notable differences among the fungi with respect to the level of correlation between the spiroketals and 1-hexanol. The spores from *Aspergillus niger* and *Penicillium glabrum* showed strong correlations between the spiroketals and 1-hexanol (0.879 – 0.986), with the chalcogran isomers having slightly larger correlations than conophthorin. The spores from *A. flavus* (atoxigenic) and *Rhizopus stolonifer* showed weak correlations (0.115 – 0.481) of the spiroketals to 1-hexanol, again with the chalcogran isomers giving better correlations and lower *P* values. *A. flavus* (toxigenic) and *A. parasiticus* did not provide evidence of a significant relationship between the noted volatiles. It should be noted that there are strong correlations among the spiroketals for all tested fungal spores (0.669 – 0.998).

Table 2 provides the correlations for the chalcogran isomers, produced by the spores on linolenic acid, to both 1-hexanol and (3*Z*)-hexen-1-ol. *Aspergillus flavus* (atoxigenic) and *A. niger* showed moderate correlations between the chalcogran isomers and (3*Z*)-hexen-1-ol (0.550 – 0.630), and the correlation between 1-hexanol and (3*Z*)-hexen-1-ol were moderate at best

(0.530 and 0.543, respectively). The remaining fungal spores showed weak correlation between the chalcogran isomers and (3*Z*)-hexen-1-ol (0.134 – 0.310), and between 1-hexanol and (3*Z*)-hexen-1-ol (0.197 – 0.318). Overall, the correlations between chalcogran isomers and 1-hexanol were weaker for the spores on linolenic then on linoleic acid, with the exception of perhaps *A. flavus* (atoxigenic). While 1-hexanol is a common volatile associated with fatty acid breakdown by fungi,[13] it should be noted that the presence of 1-hexanol has been detected in addition to the pheromone chalcogran from bark beetle (*Pityogenes quadridens*).[3] Additionally, 1-hexanol was formed along with conophthorin and both 1-hexanol and (3*Z*)-hexen-1-ol were produced when both conophthorin and chalcogran were detected from the stem extracts of two non-host angiosperms.[6]

In their 1995 report Francke et al.[3] suggested that an insect biosynthesis of chalcogran involved an unsaturated fatty acid. This idea was subsequently supported by several other reports regarding possible biosynthesis of spiroketals from insects.[2, 23, 24] In light of the evidence of the presently reported spiroketals and C6 analogues generated from spores on fatty acid we propose a possible biosynthesis of conophthorin and chalcogran from linoleic and linolenic acids, respectively (Figures 5 and 6).

Figure 5 provides the hypothesized biosynthesis of conophthorin, **1**, and the necessary ketodiol intermediate, **3**, from linoleic acid, **4**. The generation of 1-hexanol from the fungal-based lipoxygenase oxidation of linoleic via the known intermediates **5**-**7** was a good starting point for the proposed biosynthesis.[25] Note the position of the allylic alcohol in intermediate **7**. Cleavage between the vinyl and hydroxyl carbons would produce the observed volatile 1-hexanol (hypothesized steps noted with dashed reaction arrows) and intermediate **8**. Rearrangement of **8** to form the oxirane **9** followed by conjugate addition of a hydroxyl radical

would lead to the C12 compound, **10**. Known fatty acid β-oxidation would provide intermediate **11**. Subsequent oxidation at the position labeled carbon-2 to yield intermediate **12** follows a recent report on the proposed biosynthetic pathway for similar insect-derived spiroketals.[24] Decarboxylation of the terminal carboxylic acid, reduction of the ketone and the alkene, and oxidation of the alcohol at position 6 would provide the required intermediate for cyclization to form conophthorin. The order of the proposed biotransformations is not inferred and could initiate at the acid terminus or either of the alkenes.

Similarly, the production of (3*Z*)-hexen-1-ol from linolenic acid is shown in Figure 6. Since both (3*Z*)-hexen-1-ol and 1-hexanol are detected from fungal presence on linolenic acid, it is speculated that (3*Z*)-hexen-1-ol is either reduced prior to or after cleavage from the fatty acid to form 1-hexanol. The remaining biosynthesis would proceed as in Figure 5 except oxidation at position 3, instead of at position 2, in intermediate **11** would have to be accomplished. Work is underway to explore more fully the biosynthetic pathway for the production of spiroketals from fungal spores on fatty acids.

Importance of results toward other studies

The results from this study are important for several reasons. Primarily, the study demonstrates that fungal spores can generate conophthorin and chalcogran thus possibly explaining the sporadic detection of these spiroketals from almonds.[9, 10, 13, 22, 26] Anecdotal evidence from several past and present studies in our laboratories has suggested that volatile profiles for full fungal growth versus initial spore germination are different.[13-15] The unique origin of these particular spiroketals adds to the literature regarding conophthorin and chalcogran and their relationship to bark beetles. The study also demonstrates that conophthorin, an

important component of a recently reported blend of volatiles,[10] may play a critical role in the chemical communication of the navel orangeworm and its relationship to pistachio and almond orchards. The fungal spores chosen for this study have direct associations with these tree nut orchards, as do the chosen fatty acids. An investigation of orchard fungal bouquets on more complex hosts[15] is underway to further explore this interesting and complex plant/microbe interaction, as well as the possibility that these compounds can serve as markers for fungal infections in orchards.

ACKNOWLEDGEMENT

The authors thank Gloria Merrill, Sasha Flamm, and Divya Donthi (USDA-ARS) for their valuable contributions.

SUPPORTING INFORMATION

Supporting Information Available: Summary of all detected volatile products, detailed pairwise day-to-day comparisons, and a picture of the volatile collection chamber. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

(1) Zhang, Q.-H.; Tolasch, T.; Schlyter, F.; Francke, W. Enantiospecific antennal response of bark beetles to spiroacetal (*E*)-conophthorin. *J. Chem. Ecol.* **2002**, *28*, 1839-1852.

(2) Booth, Y. K.; Kitching, W.; De Voss, J. J. Biosynthesis of insect spiroacetals. *Nat. Prod. Rep.* **2009**, *26*, 490-525.

(3) Francke, W.; Bartels, J.; Meyer, H.; Schroder, F.; Kohnle, U.; Baader, E.; Vite, J. P. Semiochemicals from bark beetles: new results, remarks, and reflections. *J. Chem. Ecol.* **1995**, *21*, 1043-1063.

(4) Francke, W.; Heemann, V.; Gerken, B. 2-Ethyl-1,6-dioxaspiro[4.4]nonane, principal aggregation pheromone of *Pityogenes chalcographus* (L.). *Naturwissenschaften* **1977**, *64*, 590-591.

(5) Broberg, C. L.; Borden, J. H.; Gries, R. Antennae of *Cryptorhynchus lapathi* (Coleoptera: Curculionidae) detect two pheromone components of coniferophagous bark beetles in the stems of *Salix sitchenisis* and *Salix scouleriana* (Salicaeae). *Can. Entomol.* **2005**, *137*, 716-718.

(6) Shepherd, W. P.; Huber, D. P. W.; Seybold, S. J.; Fettig, C. J. Antennal responses of the western pine beetle, *Dendroctonus brevicomis* (Coleoptera: Curculionidae), to stem volatiles of its primary host, *Pinus ponderosa*, and nine sympatric nonhost angiosperms and conifers. *Chemoecology* **2008**, *17*, 209-221.

(7) Citron, C. A.; Rabe, P.; Dickschat, J. S. The scent of bacteria: headspace analysis for the discovery of natural products. *J. Nat. Prod.* **2012**, *75*, 1765-1776.

(8) Roitman, J. N.; Merrill, G. B.; Higbee, B. S. What attracts navel orangeworm to oviposit preferentially on wounded almonds rather than unblemished fruit? The search

for a volatile attractant. Proceedings of the 4th Fungal Genomics, 5th Fumonisin Elimination, and 17th Aflatoxin Elimination Workshop. Oct. 25-28, 2004. pg. 27.

(9) Beck, J. J.; Higbee, B. S.; Merrill, G. B.; Roitman, J. N. Comparison of volatile emissions from undamaged and mechanically damaged almonds. *J. Sci. Food Agric.* **2008**, *88*, 1363-1368.

(10) Beck, J. J.; Higbee, B. S.; Light, D. M.; Gee, W. S.; Merrill, G. B.; Hayashi, J. M. Hull split and damaged almond volatiles attract male and female navel orangeworm. *J. Agric. Food Chem.* **2012**, *60*, 8090-8096.

(11) Bayman, P.; Baker, J. L.; Mahoney, N. E. *Aspergillus* on tree nuts: incidence and associations. *Mycopathologia* **2002**, *155*, 161-169.

(12) Molyneux, R. J.; Mahoney, N.; Kim, J. H.; Campbell, B. C. Mycotoxins in edible tree nuts. *Int. J. Food Microbiol.* **2007**, *119*, 72-78.

(13) Beck, J. J.; Mahoney, N. E.; Cook, D.; Gee, W. S. Volatile analysis of ground almonds contaminated with naturally occurring fungi. *J. Agric. Food Chem.* **2011**, *59*, 6180-6187.

(14) Beck, J. J.; Merrill, G. B.; Palumbo, J. D.; O'Keeffe, T. L. Strain of *Fusarium oxysporum* isolated from almond hulls produces styrene and 7-methyl-1,3,5-cyclooctatriene as the principal volatile components. *J. Agric. Food Chem.* **2008**, *56*, 11392-11398.

(15) Beck, J. J. Addressing the complexity and diversity of agricultural plant volatiles: a call for the integration laboratory- and field-based analyses. *J. Agric. Food Chem.* **2012**, *60*, 1153-1157.

(16) Chalier, P.; Crouzet, J. Production of volatile components by *Pencillium roqueforti* cultivated in the presence of soya bean oil. *Flavour Frag. J.* **1993**, *8*, 43-49.

(17) Park, O. -J.; Holland, H. L.; Khan, J. A.; Vulfson, E. N. Production of flavour ketones in aqueous-organic two-phase systems by using free and microencapsulated fungal spores as biocatalysts. *Enzyme Microb. Tech.* **2000**, *26*, 235-242.

(18) Clarke, J. A.; Brar, G. S.; Procopiou, J. Fatty acid, carbohydrate and amino acid composition of pistachio (*Pistacia vera*) kernels. *Qual. Plant.* **1976**, *25*, 219-225.

(19) Sathe, S. K.; Seeram, N. P.; Kshirsagar, H. H.; Heber, D.; Lapsley, K. A. Fatty acid composition of California grown almonds. *J. Food Sci.* **2008**, *73*, C607-614.

(20) Dantigny, P.; Nanguy, S. P.-M. Significance of the physiological state of fungal spores. *Int. J. Food Microbiol*. **2009**, *134*, 16-20.

(21) Gougouli, M.; Koutsoumanis, K. P. Modeling germination of fungal spores at constant and fluctuating temperature conditions. *Int. J. Food Microbiol*. **2012**, *152*, 153-161.

(22) Beck, J. J.; Merrill, G. B.; Higbee, B. S.; Light, D. M.; Gee, W. S. *In situ* seasonal study of the volatile production of almonds (*Prunus dulcis*) var. 'Nonpareil' and relationship to navel orangeworm. *J. Agric. Food Chem.* **2009**, *57*, 3749-3753.

(23) Seybold, S. J.; Bohlmann, J.; Raffa, K. F. Biosynthesis of coniferophagous bark beetle pheromones and conifer isoprenoids: evolutionary perspective and synthesis. *Can. Entomol.* **2000**, *132*, 697-753.

(24) Booth, Y. K.; Kitching, W.; De Voss, J. J. Biosynthesis of the spiroacetal suite in *Bactrocera tryoni*. *ChemBioChem* **2011**, *12*, 155-172.

(25) Gardner, H. W. Recent investigations into the lipoxygenase pathway of plants. *Biochim. Biophys. Acta* **1991**, *1084*, 221-239

406 (26) Beck, J. J.; Higbee, B. S.; Gee, W. S.; Dragull, K. Ambient orchard volatiles from
407 California almonds. *Phytochem. Lett.* **2011**, *4*, 199-202.

408

409 Research was conducted under USDA-ARS CRIS Project 5325-42000-037-00D, TFCA 5325-
410 42000-037-07 with the California Pistachio Research Board, TFCA 5325-42000-037-05 with the
411 Almond Board of California, and portions of 5325-42000-037-13 with the California Department
412 of Food and Agriculture.

413

414 **FIGURE CAPTIONS**

415 **Figure 1.** Chemical structures showing the relative *cis*/*trans* configurations, possible
416 stereoisomers, and pertinent numbering for conophthorin, 7-methyl-1,6-dioxaspiro[4.5]decane,
417 **1**, and chalcogran, 2-ethyl-1,6-dioxaspiro[4.4]nonane, **2**.

418

419 **Figure 2.** Means of sums of spiroketal production over the 13 days of volatile collection for
420 investigated spores on (A) linoleic acid and (B) linolenic acid. Same case letters above error bars
421 indicate statistical significance between fungal spores. Error bars show standard error mean.

422

423 **Figure 3.** Spiroketal production by fungal spores on linoleic acid at specific volatile collection
424 time points (d = 0, 1, 4, 6, 8, 11, and 13). Error bars show standard error mean for the spiroketal
425 production within each fungal spore.

426

427 **Figure 4.** (A) Chalcogran 1 and (B) chalcogran 2 production by fungal spores on linolenic acid
428 at specific volatile collection time points (d = 0, 1, 4, 6, 8, 11, and 13). Error bars show standard
429 error mean for the chalcogran production within each fungal spore.

430

431 **Figure 5.** Hypothesized biosynthesis of 1-hexanol and conophthorin from fungal spores on
432 linoleic acid.

433

434 **Figure 6.** Hypothesized biosynthesis of (3*Z*)-hexen-1-ol from fungal spores on linolenic acid.

435

436

Table 1. Pearson correlations[a] for spiroketals and 1-hexanol produced by noted fungal spores on linoleic acid.

		conophthorin	chalcogran 2	1-hexanol
***A. flavus* (atoxigenic)**	**conophthorin**			0.437
				(0.048)
	chalcogran 1	0.885	0.984	0.682
		(0.000)	(0.000)	(0.001)
	chalcogran 2	0.928		0.654
		(0.000)		(0.001)
***A. flavus* (toxigenic)**	**conophthorin**			0.115
				(0.619)
	chalcogran 1	0.827	0.982	0.385
		(0.000)	(0.000)	(0.085)
	chalcogran 2	0.833		0.299
		(0.000)		(0.188)
A. niger	**conophthorin**			0.879
				(0.000)
	chalcogran 1	0.909	0.980	0.953
		(0.000)	(0.000)	(0.000)
	chalcogran 2	0.966		0.947
		(0.000)		(0.000)
A. parasiticus	**conophthorin**			0.276
				(0.226)
	chalcogran 1	0.902	0.992	0.405
		(0.000)	(0.000)	(0.069)
	chalcogran 2	0.920		0.406
		(0.000)		(0.068)
Penicillium glabrum	**conophthorin**			0.965
				(0.000)
	chalcogran 1	0.990	0.998	0.986
		(0.000)	(0.000)	(0.000)
	chalcogran 2	0.992		0.985
		(0.000)		(0.000)
Rhizopus stolonifer	**conophthorin**			0.246
				(0.295)
	chalcogran 1	0.669	0.956	0.450
		(0.001)	(0.000)	(0.041)
	chalcogran 2	0.691		0.481
		(0.001)		(0.027)

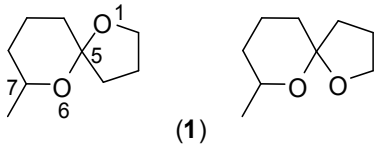
[a] Pairs of variables with positive correlation coefficients and with *P* values that are below 0.050 tend to increase together. For pairs with *P* values greater than 0.050, there is no significant relationship between the two variables. Corresponding *P* values are shown in parentheses.

Table 2. Pearson correlations[a] for spiroketals and green leaf volatiles produced by noted fungal spores on linolenic acid.

		chalcogran 2	1-hexanol	(3*Z*)-hexen-1-ol
***A. flavus* (atoxigenic)**	**chalcogran 1**	0.992	0.775	0.630
		(0.000)	(0.000)	(0.003)
	chalcogran 2		0.781	0.611
			(0.000)	(0.003)
	1-hexanol			0.530
				(0.014)
***A. flavus* (toxigenic)**	**chalcogran 1**	0.992	0.374	0.310
		(0.000)	(0.095)	(0.172)
	chalcogran 2		0.336	0.257
			(0.136)	(0.260)
	1-hexanol			0.318
				(0.160)
A. niger	**chalcogran 1**	0.992	0.472	0.603
		(0.000)	(0.031)	(0.004)
	chalcogran 2		0.436	0.550
			(0.048)	(0.010)
	1-hexanol			0.543
				(0.011)
A. parasiticus	**chalcogran 1**	0.994	-	0.238
		(0.000)	-	(0.299)
	chalcogran 2		-	0.195
			-	(0.397)
	1-hexanol			-
				-
Penicillium glabrum	**chalcogran 1**	0.995	0.423	0.166
		(0.000)	(0.071)	(0.471)
	chalcogran 2		0.422	0.134
			(0.072)	(0.561)
	1-hexanol			0.197
				(0.420)

[a] Pairs of variables with positive correlation coefficients and with *P* values that are below 0.050 tend to increase together. For pairs with *P* values greater than 0.050, there is no significant relationship between the two variables. Corresponding *P* values are shown in parentheses.

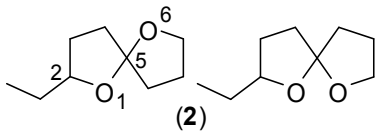
Figure 1



(**1**)

(*E*)-(5*R*,7*R*) (*Z*)-(5*S*,7*R*)

(*E*)-(5*S*,7*S*) (*Z*)-(5*R*,7*S*)



(**2**)

(*E*)-(2*S*,5*R*) (*Z*)-(2*R*,5*R*)

(*E*)-(2*R*,5*S*) (*Z*)-(2*S*,5*S*)

Figure 2.

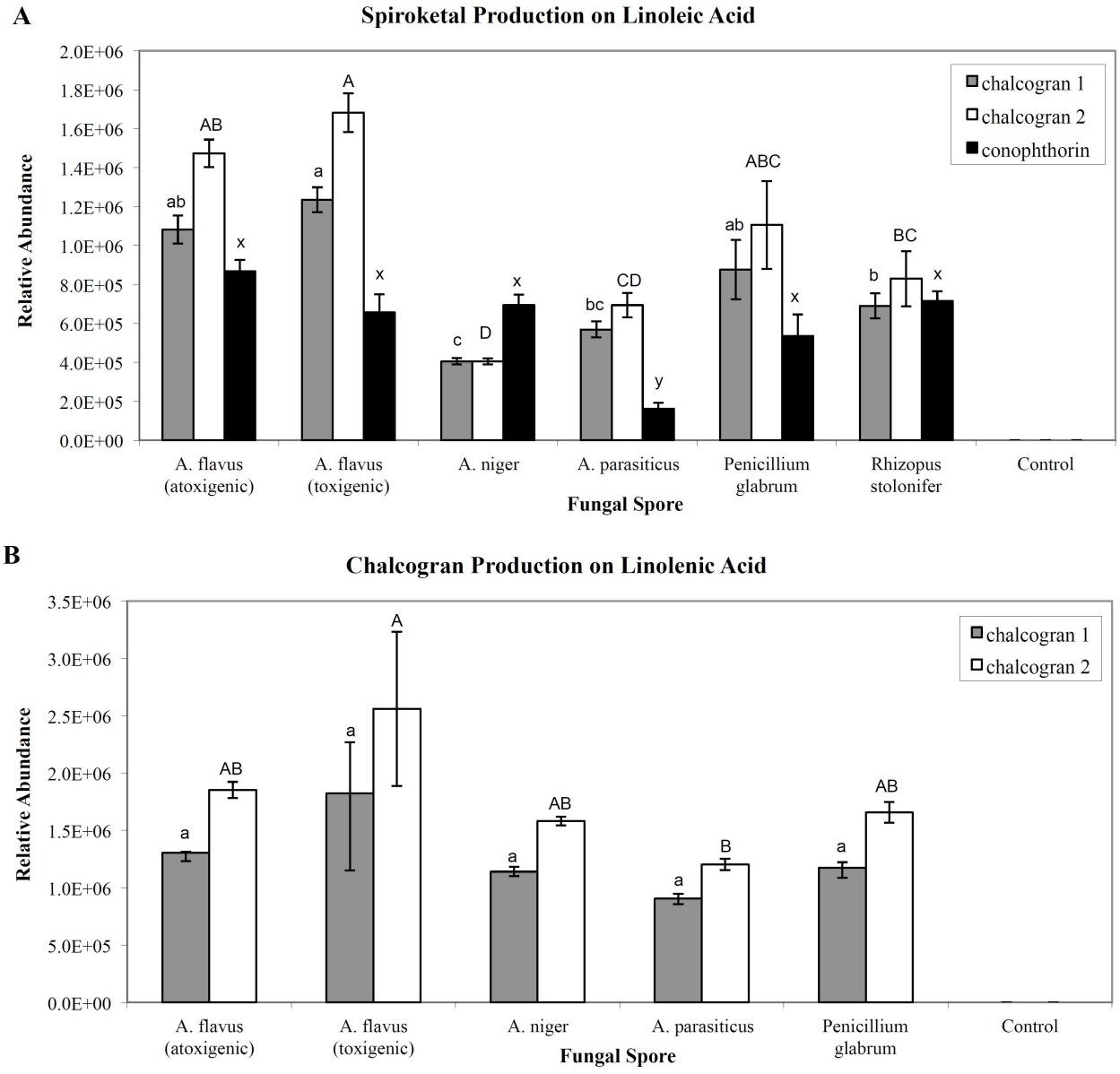

A
Spiroketal Production on Linoleic Acid
Relative Abundance
2.0E+06
1.8E+06
1.6E+06
1.4E+06
1.2E+06
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
chalcogran 1
chalcogran 2
conophthorin
ab
AB
x
a
A
x
c
D
x
bc
CD
y
ab
ABC
x
b
BC
x
A. flavus (atoxigenic)
A. flavus (toxigenic)
A. niger
A. parasiticus
Penicillium glabrum
Rhizopus stolonifer
Control
Fungal Spore
B
Chalcogran Production on Linolenic Acid
Relative Abundance
3.5E+06
3.0E+06
2.5E+06
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
chalcogran 1
chalcogran 2
a
AB
a
A
a
AB
a
B
a
AB
A. flavus (atoxigenic)
A. flavus (toxigenic)
A. niger
A. parasiticus
Penicillium glabrum
Control
Fungal Spore

Figure 3.

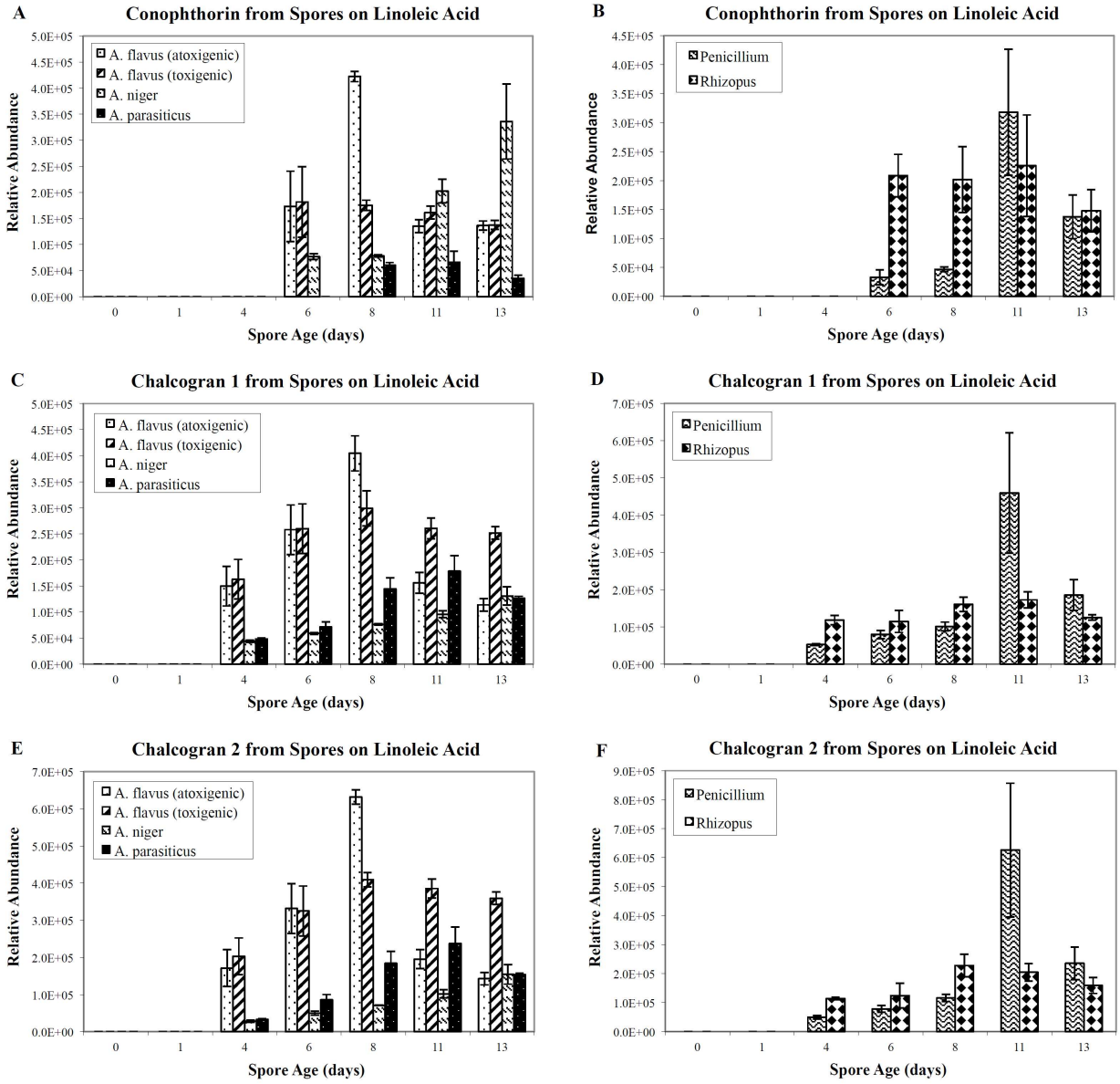

A
Conophthorin from Spores on Linoleic Acid
A. flavus (atoxigenic)
A. flavus (toxigenic)
A. niger
A. parasiticus
Relative Abundance
Spore Age (days)
B
Conophthorin from Spores on Linoleic Acid
Penicillium
Rhizopus
Relative Abundance
Spore Age (days)
C
Chalcogran 1 from Spores on Linoleic Acid
A. flavus (atoxigenic)
A. flavus (toxigenic)
A. niger
A. parasiticus
Relative Abundance
Spore Age (days)
D
Chalcogran 1 from Spores on Linoleic Acid
Penicillium
Rhizopus
Relative Abundance
Spore Age (days)
E
Chalcogran 2 from Spores on Linoleic Acid
A. flavus (atoxigenic)
A. flavus (toxigenic)
A. niger
A. parasiticus
Relative Abundance
Spore Age (days)
F
Chalcogran 2 from Spores on Linoleic Acid
Penicillium
Rhizopus
Relative Abundance
Spore Age (days)

Figure 4.

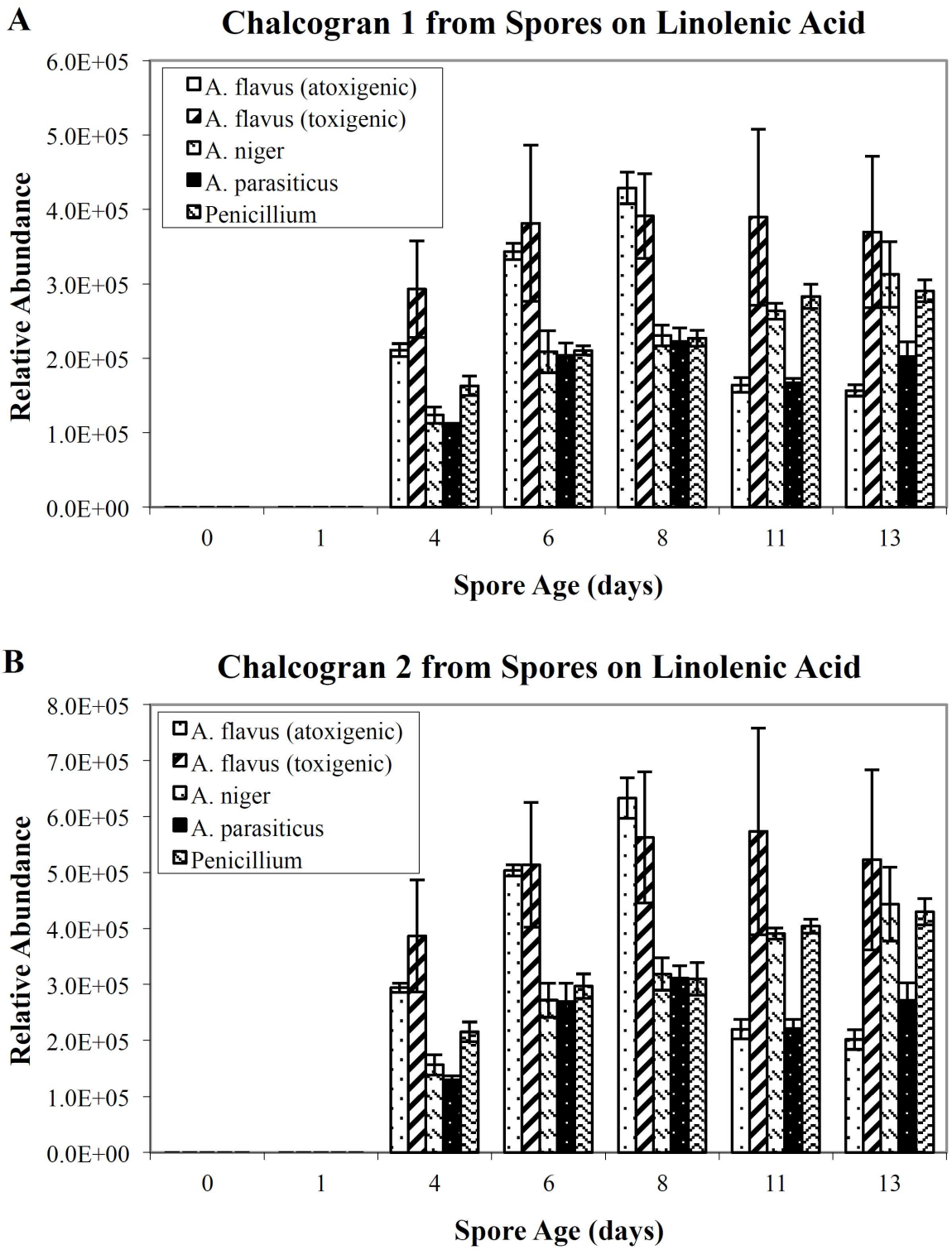

A
Chalcogran 1 from Spores on Linolenic Acid
Relative Abundance
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
A. flavus (atoxigenic)
A. flavus (toxigenic)
A. niger
A. parasiticus
Penicillium
0
1
4
6
8
11
13
Spore Age (days)
B
Chalcogran 2 from Spores on Linolenic Acid
Relative Abundance
8.0E+05
7.0E+05
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
A. flavus (atoxigenic)
A. flavus (toxigenic)
A. niger
A. parasiticus
Penicillium
0
1
4
6
8
11
13
Spore Age (days)

Figure 5.

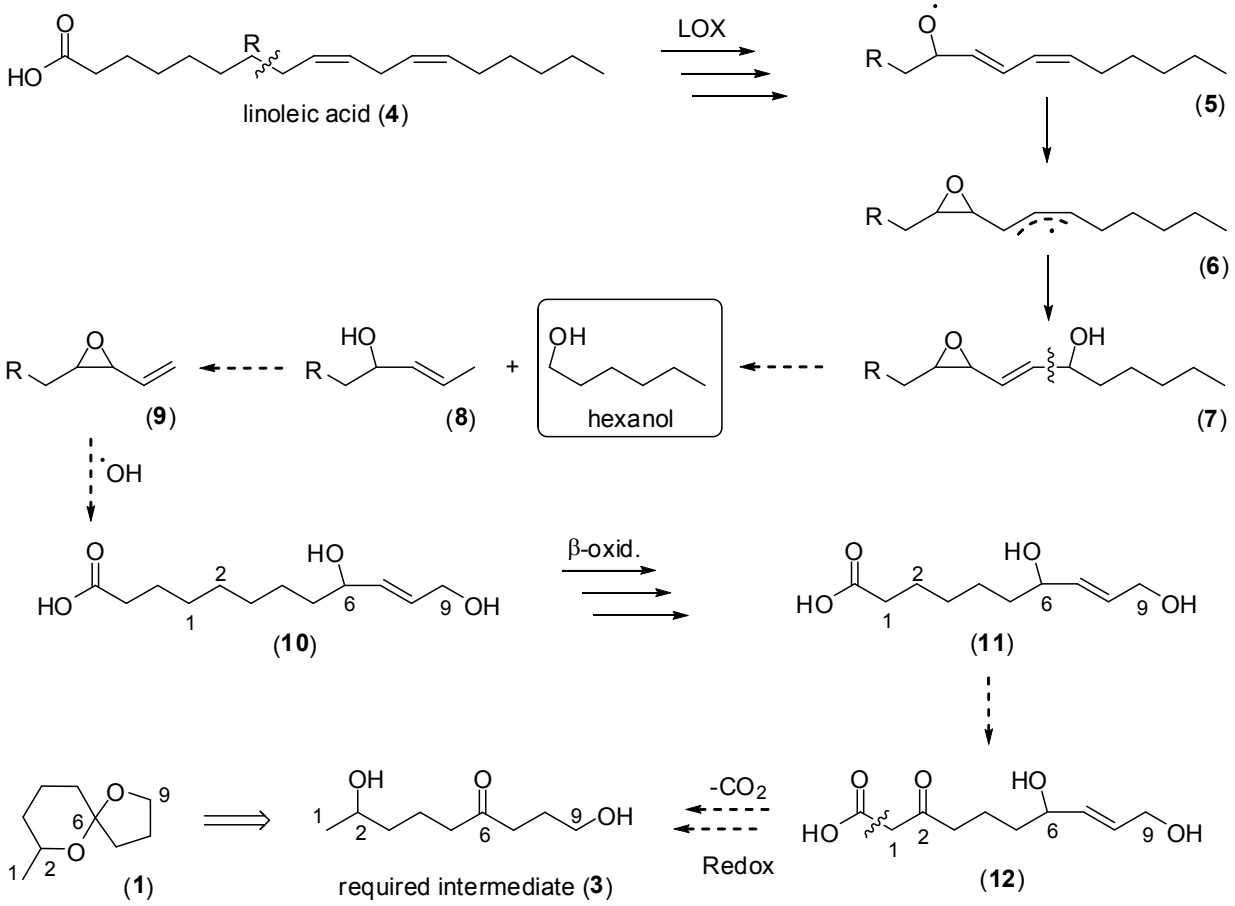
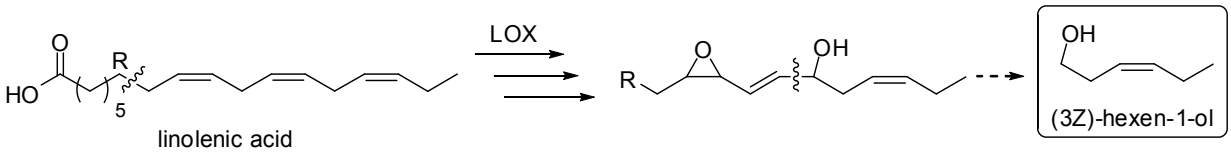

LOX
linoleic acid (4)
(5)
(6)
(7)
(8)
(9)
hexanol
β-oxid.
(10)
(11)
(12)
(1)
required intermediate (3)
-CO2
Redox

Figure 6.



TOC GRAPHIC

Beck, Mahoney, *et al*.

Generation of the Volatile Spiroketals Conophthorin and Chalcogran by Fungal Spores on Polyunsaturated Fatty Acids Common to Almonds and Pistachios